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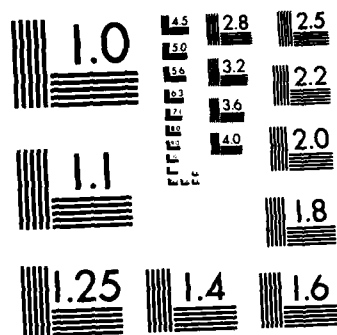
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MICROBIAL GROWTH AT ULTRASLOW RATES:
REGULATION AND GENETIC STABILITY

FINAL REPORT

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PROFESSOR OF MICROBIOLOGY
MARCH 1983

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A fermentor system has been developed to study bacterial growth from doubling times of 0.5 to 300 h. The studies show that specific growth rate (μ), molar growth yield (Y), and nutrient levels cannot be related by any continuous mathematical expression. Instead, there are 3 growth rate ranges in bacterial growth containing 3 metabolic phases or domains -- exploitative, adaptive, and conservative -- which are sharply separated from each other by two gaps, or non-existent growth rate ranges, between doubling times of 15-20 h and between 80 and 125 h.		

FOREWORD

During 1972-73, I was a Visiting Investigator in the laboratory of Dr. Gerald Silverman at Natick Laboratories, and worked with him on the problem of generation of toxins by bacteria growing in foodstuffs (Chesbro et al., 1976). After returning to my permanent position at the University of New Hampshire and reflecting on the collaborative work with Dr. Silverman, I became increasingly aware of the fact that there was relatively little known about microbial behavior at slow growth rates in contrast to the situation for microbial behavior at fast growth rates, despite the fact that there are many important natural situations where low, rather than high, growth rates should be the usual condition. For instance, whenever a bacterial symbiont or pathogen is in equilibrium or near equilibrium with its host, as in the rhizobium/legume situation or in chronic infections, the bacterial population must be static in size, or only increasing slowly. In general, bacteria inhabiting nutritionally impoverished environments must be growing slowly, even when attached to nutrient-concentrating surfaces, since their rate of nutrient uptake will be too slow to maintain rapid growth. Often in industrial applications slow growth with rapid dissimilation of substrate is desired to maximize production of some secondary metabolite -- a secreted enzyme or antibiotic, for example -- and in waste treatment, this is always the ideal operating situation, after startup, since it means the slowest rate of biomass accumulation as sludge. Further, the coordination and expression of genetic and mutational processes at slow growth rates is intrinsically interesting in addition to the bearing it has on senescence and aging.

The main reason for the presence of a large volume of information on rapid bacterial growth is the existence of an excellent device, the chemostat, for producing populations of microorganisms growing under closely known conditions of cultivation at exactly known, constant, specific growth rates, all of which are under the experimenter's control. However, the range of specific growth rates that can be reliably reproduced in chemostats has a sharp lower limit at specific growth rates (μ) between 0.1 and 0.05 h⁻¹ corresponding to doubling times of 7 to 14 h. This is caused by the increasingly pulsatile nature of chemostat operation in this region, associated with the mechanical properties of the device as it is usually constructed. While seemingly this is a trivial problem, overcoming it represents a serious

engineering challenge. Consequently, all studies of growth using the chemostat of which I am aware, save only a few, do not go beyond this range.

What seemed to be needed then, was a device that, like the chemostat, allowed growing bacterial populations with precisely reproducible and controllable specific growth rates, but which did not have the lower operational limit of the chemostat.

Such a device seemed implicit in descriptions made by Schultz and Gerhardt (1969) of microbial growth in dialysis culture, where the microbial population is retained within a dialysis membrane across which fresh nutrients diffuse into, and spent medium diffuses out of, the culture. Their analysis indicated that μ for such a culture falls along a continuous gradient asymptotically approaching zero as the growing population diverts a continuously larger fraction of the influent energy substrate to maintenance metabolism that does not produce new biomass. The analysis depended on the equations derived by Pirt (1965) defining the effect on μ of this partition of energy substrate between maintenance and growth metabolism. Pirt's equations in turn are transformations of Monod's (1942) description of the relation of μ to nutrient concentration in the growth medium.

Actually, several devices are implied by these analyses, all of which should produce populations of very slowly growing bacteria, with specific growth rates well below 0.1 to 0.05 h^{-1} . In 1976, Drs. Thomas Evans and Robin Eifert, then Ph.D. candidates in the Department of Microbiology at the University of New Hampshire, and I built the first of these in the form of a recycling fermentor in which the microbial biomass is completely retained in the growth chamber while fresh medium is brought to it, and spent medium carried away, at controlled rates.

Within the first months of operation, with Escherichia coli as the test organism, it was apparent that the predictions of Schultz and Gerhardt were not fulfilled, nor those of Pirt, nor ultimately those of Monod. In the subsequent seven years, primarily with the collaboration of Dr. Michael Arbige, then a Ph.D. candidate, and with assistance from Mr. Joel Ackerman and Ms. Melissa Rochkind, two other graduate students, these observations have been extended to 12 strains of E. coli and one strain each of Yersinia pestis and Bacillus polymyxa. In collaboration with Drs. Henk van Verseveld and Adrien Southamer of the Microbiology Department of the Free University of Amsterdam, they have been further extended to Paracoccus denitrificans.

As a result of these observations across this taxonomic range of eubacteria, and the understanding we have gained of underlying biochemical and genetic machineries, it is clear that any treatment of bacterial growth which starts with the assumption that there is continuous function relating μ to rate of substrate uptake -- such as Pirt's, Monod's, and all others of which I am aware -- starts with an assumption that is wrong.

Contrary to an intuitive perception that such a continuity should exist, from which these treatments are derived, there are at least two distinct ranges of μ which are nonexistent, or exist so transiently as to be nonexistent for practical purposes, so that there are gaps in a plot of μ versus substrate uptake rate. And for the ranges of μ which do exist, between these gaps, the equations developed from assuming continuity are invalid or describe the relationships between μ , the molar growth yield, Y , and substrate uptake in a trivial way.

The first gap occurs just below the range of chemostat operations, at about a μ of 0.06 h^{-1} , the second where μ is about 0.012 h^{-1} . Although we do not know yet what the first gap is due to, the second is clearly due to the activity of the regulatory system of nucleotides centered on guanosine tetra phosphate (ppGpp) generally called the stringent response (Arbige and Chesbro, 1982a), which is common to eubacteria and whose effects on μ and Y , in fact, made the Monod-type equations invalid as soon as they were elucidated to the level reached by 1976, whether or not we had ever performed our experiments. There is yet no apparent general realization of this among biologists, however.

The significance of these findings about the basic equations relating bacterial growth rates and yields to substrate levels is that any studies to date in biochemical engineering, on biological waste water treatment, or in microbial bioenergetics are incorrect to the extent that they depend on these equations, with the degree of incorrectness increasing by quantum jumps across each of the two gaps thus far located.

A truer approximation of the relationship of microbial growth to nutrient levels is now available that takes into account the fact that microorganisms must make major changes in their metabolic patterns at specific growth rates to realign sequences in the biosynthesis of important macromolecules and cell structures, and that these changes are basically independent of nutrient levels although they are normally coupled to them. With this

understanding it should be possible to improve our understanding and control of microbial growth whether in biotechnological, clinical, ecological, or basic applications.

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REPORT OF PROJECT FINDINGS

a. Statement of problem

The initial problem was to produce a device that would reproducibly carry the specific growth rate of a bacterial culture to lower levels than those that can be reached reliably in a chemostat, but which had the chemostat's flexibility of operation and ease of instrumentation. This would permit studying the genetic, metabolic, and regulatory behavior of bacteria at growth rates infrequently studied, but which are apt to occur commonly in a variety of natural situations and biotechnological applications (see "Foreword"), and could provide insight into how mutation and repair of genetic damage was affected by growth at these low, senescent rates.

As noted in the "Foreword", after building and operating such a device, it quickly became apparent that the accepted equations relating μ , Y , and nutrient levels were derived from the incorrect premise that there is a continuous function relating these parameters. The problem then expanded to deriving a truer approximation to the relationship between these growth parameters. Thus, we sought specifically:

- 1) to find if there was a pattern of growth behavior at slow rates common among eubacteria;
- 2) to identify the genetic/biochemical mechanisms whose activity invalidated the presumed relationship between μ , y , and nutrient level;
- 3) to describe the behavior of the site-fixed pathogens Yersinia pestis and enterotoxigenic E. coli at slow growth rates;
- 4) to measure gene expression and gene repair and enzyme (protein) stability in slowly growing cells;
- 5) to determine if bacteriophage could attach and multiply in slowly-growing, energy depleted hosts.

b. Important results

1. The Basic Growth Pattern of Eubacteria

In the recycling fermentor (Fig. 1) the growth pattern shown in Figure 2 has been observed with 12 strains of E. coli, and 1 strain each of Bacillus polymyxa, Yersinia pestis, and Paracoccus denitrificans. The 3 phase pattern is observed growing the 3 facultative bacteria anaerobically (it was found that Y. pestis, although defined as facultative is, in fact, microaerophilic) and the aerobic bacterium P. denitrificans with oxygen.

It is also observed for E. coli growing aerobically or anaerobically in fed batch culture (see Appendix, summary of manuscript by van Versevelde, Chesbro and Stouthamer) and the defining features of the pattern can be seen when the behavior of P. denitrificans, carbon/energy downshifted in a chemostat, is computer analyzed (see Appendix, Abstract of paper presented by Braster et al., in Dec. 1982 in Apeldoorn, The Netherlands).

Thus, the pattern is common to eubacteria, independent of the mechanical features of the growth system, and applies to both aerobic and anaerobic energy metabolism.

Equations of the Monod-type, predicated on a continuous relationship between μ and nutrient uptake rate, would require the growth curve to asymptote to a maximum and do not predict the 2 abrupt breaks in μ observed.

These are seen more clearly in Figure 2, where the growth pattern of 4 E. coli rel mutants and the wild-type parent are shown, and the growth pattern of the parent has been resolved in terms of μ and doubling time (T_D). The two discontinuities are clearly evident at $T_D = 15$ h and $T_D = 80$ h. The T_D values (and corresponding μ values) between 15 - 20 h and 80 - 125 h effectively do not exist. At these 2 points, Y drops abruptly by about 30%, but has a constant value within each of the 3 phases that lie between the discontinuities. This again contradicts Monod-type expectations. We have analyzed this in detail (Chesbro et al., 1979; Arbige et al., 1982a; Arbige et al., 1982b).

Briefly, in the fermentor there is a continuous, progressive carbon-energy shortfall per cell, carrying down μ . Within the cell, this dwindling carbon-energy uptake is, in essence, metered by the products of the rel system genes relA, relX(S), and spoT. When the T_D has fallen to 15 h, these

gene products, which are the enzymes for the synthesis and degradation of the family of regulatory nucleotides centered on guanosine tetraphosphate (ppGpp), are progressively activated and inactivated, respectively, so that throughout the second phase (T_D goes from 20 h to 80 h) the cellular concentration of ppGpp is rising. At $T_D = 80$ h, ppGpp has reached the level at which it interdicts the synthesis of ribosomes and the elongation of proteins, producing the abrupt drop in μ and Y characterizing the second discontinuity, and the corresponding jump in T_D to 125 h.

The reason that this regulatory mechanism operates at this particular value of μ in eubacteria is that, if it does not, inherent kinetic features of the steps in protein synthesis dictate that mistranslation will increase rapidly.

The drop in Y at the first discontinuity is proportional to that at the second, arguing that the cell must divert an equally substantial fraction of its available energy to correcting some incipient imbalance in basic biosynthetic processes developing at $T_D = 15$ h. However, we have not identified what this imbalance is, nor how it is corrected. We do know that cAMP suddenly rises to a maximum in the cell at this point, and the cost of manufacturing this nucleotide must account for some part of the drop in y .

It is clear that there is not a continuous spectrum of growth rates in eubacteria with a corresponding continuum of metabolic states, and this would be true simply from what has been known about stringent (ppGpp) regulation since 1976, whether or not experiments of the sort we have performed were done to demonstrate this.

Instead, there are three distinct metabolic/growth rate domains, each with characterizing features, sharply separated from each other by the two gaps caused in one case certainly, and in the other case probably, by the abrupt elevation and activity of regulatory nucleotides whose synthesis is keyed to the cell's rate of nutrient uptake.

These three domains, corresponding to growth phases 1, 2 and 3 in the recycling fermentor, and their distinguishing features are described in Table 1.

2. Adaptive Enzyme and Enterotoxin Expression in the Domains of Slow Growth

When bacteria are in the two lower growth rate domains, both cAMP and ppGpp levels are elevated in the cell. Thus, expression of operons and regulons under the control of cAMP is maximal.

Figure 3 shows the continuous induction of beta galactosidase in the lowest domain (phase 3) in the fermentor. The enzyme's appearance as a result of an inductive pulse in the middle domain (phase 2), its subsequent disappearance in the mid and lowest domains, and after an energy shift up are shown in Figure 4.

Changes in the specific activity of beta galactosidase and tryptophanase in cells of the enterotoxigenic *E. coli* strain H10407 exposed continuously to the appropriate inducer throughout passage through the three domains is shown in Figures 5 and 6. Production of beta galactosidase activity is restrained by stringency (phase/domain 3) in the same way as general cell biomass production is restrained. Production of tryptophanase activity, after reaching a maximum in phase/domain 2, is thereafter slightly disfavored against production of general biomass without reference to the shift to stringency.

Secretion of the heat labile (LT) enterotoxin specific activity is favored in the first domain, less favored in the second domain and when the culture is returned from the third to the second domain by a carbon energy upshift, but clearly is favored over general biomass synthesis in the third phase/domain of stringent regulation. Its favored synthesis in this phase is lost when the culture is returned to the second domain by a carbon-energy upshift.

The response of this toxin's production to the onset of the third domain is even clearer in a second strain 711P307 (Fig. 7), and it is apparent that production of the proteolytically nicked, active toxin is the favored form. In this strain, however, an upshift strongly favors appearance of the nicked toxin; that is, the nicking process is apparently energy dependent.

Both cAMP and ppGpp play roles in this toxin's production. Figure 8 shows the reduced production of toxin in a *crp* mutant of 711P307 which lacks the functional receptor protein necessary for expression of cAMP effects.

Overall, the toxin has been shown to be affected by both cAMP and ppGpp regulation, positively in both cases, and its activation energy dependent, although strain differences in behavior exist. The toxin's manufacture is thus different than that of either of the two inducible enzymes, whose production can be obtained in the two slower growth domains, since cAMP is present in both, but whose synthesis relative to general biomass synthesis is not favored.

3. Activity of the S.O.S. System of DNA Repair (the rec-lex Gene System) in the Lowest Domain.

The major route by which chromosome lesions are fixed as mutations is via the activity of the product of the recA gene, protein X, which repairs double strand damage to the chromosome without retaining fidelity to the original nucleotide sequence. This protein is also a protease.

How this system functions in the slowly growing, stringently regulated cells of the third phase/domain was studied in two strains of E. coli. In strain ATCC33311, a modified lambda phage genome containing the betagalactosidase locus has been introduced into the bacterial chromosome, thus when the recA product is active in these cells, it cleaves the lambda repressor causing the production of beta galactosidase. In strain NF161(lambda) the unmodified phage is present, and active recA protein cleaves the phage repressor to initiate the lytic cycle. Two agents were used to affect the chromosome and activate the recA product: nalidixic acid which interferes with chromosome replication at the replicating fork and MNNG which causes breaks in both replicating and non-replicating chromosomes.

Figure 9 shows changes in cell levels of betagalactosidase and tryptophanase pulse induced in the unlysogenized NF161 strain in the second domain with administration of a pulse of nalidixic acid in the third domain. The antibiotic pulse washes out of the fermentor with first order kinetics, its level falling below that needed to inhibit the culture after 15 h. After this time, the culture resumes growing.

Following the nalidixic pulse, there is an immediate acceleration in the disappearance of tryptophanase which ceases after 10 h. The disappearance of galactosidase activity accelerates only as the accelerated disappearance of tryptophanase slows.

In ATCC33311, tryptophanase was pulse induced in the same way, then the culture was pulsed in the third domain with nalidixic acid (Fig. 10). The accelerated disappearance of tryptophanase is concurrent with the recA activation of galactosidase synthesis, and this latter synthesis becomes linear concurrent with the deceleration of tryptophanase disappearance.

When NF161(λ), the lysogenized culture of NF161, was nalidixate pulsed in the third domain (Fig. 11), there was a 5 h delay before cell lysis and phage appearance occurred. About 40% of the cells lysed. The balance of the cells resumed growth as the antibiotic pulse washed from the fermentor. Application of MNNG to these cells resulted in lysis and release of phage from over 90% of the cells within 3 h.

From these results, it can be concluded that only 40% of the cells in the third domain have an actively replicating chromosome. These cells produce an active recA product upon "jamming" of the replicating fork with nalidixic acid. RecA product synthesis is immediate gauged from the prompt production of the λ -coupled galactosidase after administration of nalidixic acid, and its proteolytic activity seems likely to be responsible for the accelerated destruction of tryptophanase.

The lag between the nalidixate pulse and the acceleration in the destruction of the wild-type galactosidase is puzzling. Its onset corresponds to the time at which the accelerated destruction of tryptophanase stops, and in the lysogenized strain, lysis and phage appear. The latter phenomenon, a 6 h lag between the appearance of recA product activity and the appearance of phage, represents the intrinsic effect upon phage assembly of the third domain. The galactosidase destruction, therefore, seems to be due to some sequence initiated at the nalidixate pulse that then required 5-6 h of biosynthetic activity at the pace of the third domain to reach the stage at which galactosidase destruction was commenced.

In summary, the activity of the rec gene and its product is not directly under the control of ppGpp and stringency. However, in the third domain only a fraction of the cells in the population have an actively replicating chromosome, so that mutational agents acting at the replicating fork will necessarily activate the rec system only in that cell fraction. Virus assembly on the other hand is under control of the stringent regulation that is the dominant feature of the third domain.

It is also clear that a gene locus, once derepressed by the action of the recA gene product, remains derepressed for an indefinitely long time in the third domain (note the continuing synthesis of the lambda-coupled galactosidase in Fig. 10).

C. Publications and Technical Reports

Chesbro, W.R., T. Evans, and R. Eifert. 1979. Very slow growth of Escherichia coli. J. Bacteriol. 139:625-638.

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Arbige, M. and W. Chesbro. 1982. Very slow growth of Bacillus polymyxa stringent response and maintenance energy. Arch. Microbiol. 132:338-344.

D. Participating Personnel and Advanced Degrees.

	Degree
Joel Ackerman	M.S.
Michael Arbige	Ph.D.
Thomas Evans	Ph.D.
Robin Eifert	Ph.D.

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APPENDIX

VERY SLOW GROWTH
OF Paracoccus denitrificans
IN A RECYCLING FERMENTOR

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Chemostat cultures of Paracoccus denitrificans were shifted to recycling conditions with 100% cell recovery. After the shift, two different growth phases could be recognized.

Phase 1, the chemostat experiment, is characterized by steady state growth with a molecular growth yield on the used carbon-source mannitol (Y_{mann}) of about 85. Phase 2, immediately after the shift, is characterized by a linear increase in biomass and a Y_{mann} of about 55 and phase 3 also shows a linear increase in biomass but at a lower rate together with a lower Y_{mann} of about 25.

Growth of Pa. denitrificans shifts from phase 2 to phase 3 after 62 hours at a specific growth rate (μ) of 0.01. As a consequence of two linear phases μ drops from 0.01 to 0.003 at the time of this shift. These experiments strongly indicate a stringent response in phase 3 as has been described before in E. coli (Chesbro et al., 1979).

The disadvantage of a recycling fermentor is that it takes 62 hours before a stringent response is obvious. In a Wash-Out experiment, in which the substrate provision rate (SPR) to a chemostat is lowered from 1.1 mM mannitol/1.h to 0.09 mM, immediately after the start of the wash-out growth becomes stringent. Measured dry weights (g biomass/l) can be fitted to Eq.1, which describes the behavior of a wash-out culture.

$$x(t) = \frac{(SPR) \cdot Y_{mann}}{D} + \left(x(0) - \frac{(SPR) \cdot Y_{mann}}{D} \right) \cdot e^{-Dt} \quad (1)$$

in which $x(t)$ is the dry weight at time= t , $x(0)$ is the starting dry weight and D is the dilution rate. The wash-out experiment can be described with the same Y_{mann} values of phase 1 and 2 of the recycling experiment, except that in the first 8 hours after the start, the decrease in dry weight is faster than the calculated wash-out for Y_{mann}=0. Furthermore, this method is not sensitive enough for an exact determination of Y_{mann} of phase 3.

Under stringent response situations (μ is lower than 0.01) the cellular ppGpp level increases as has been found before in E. coli (Arbige and Chesbro, 1982).

Results presented shed doubt on the validity of the Pirt-equation (Pirt, 1975). A possible explanation of our results is the occurrence of 3 distinct growth situations:

- (1) growth under stringent response at a μ lower than 0.01 and $Y_{mann}=25$.
- (2) an intermediate situation (phase 2 in recycling experiments at μ values between 0.01 and 0.05, $Y_{mann}=55$.
- (3) growth with high efficiency at μ higher than 0.05, $Y_{mann}=85$.

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Three Growth Rate Domains in Eubacteria Determined
by Nutrient Availability

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-Abstract-

Growth of Escherichia coli and Paracoccus denitrificans has been studied in chemostat, fed batch, and recycling fermentor modes under carbon energy limitation. Two abrupt drops in the molar growth yield, Y , have been found that occur over relatively short ranges in the value of μ . After the first of these, at a doubling time of 14 h, Y becomes constant and independent of μ until the second discontinuity appears at a doubling time of about 85 h, after which Y again becomes constant. The second discontinuity is associated in Paracoccus with elevated levels of guanosine tetraphosphate (ppGpp) that impose stringent regulation, as had been found previously with Bacillus and Escherichia species. It is thus likely that the stringent response generally occurs in bacteria in vivo at a doubling time of about 85 h. The cause of the first discontinuity is unknown, but after it is reached Pirt-type calculations relating μ , Y , and maintenance energy demand are no longer valid. Three regions of bacterial growth rates are defined by the study, corresponding to doubling times of 0.5 to 14 h, 20 - 85 h, and longer than 130 h. Some growth behavior in each region is unique to that region.

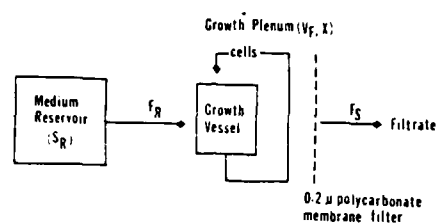


Fig. 1. Schematic of recycling fermentor

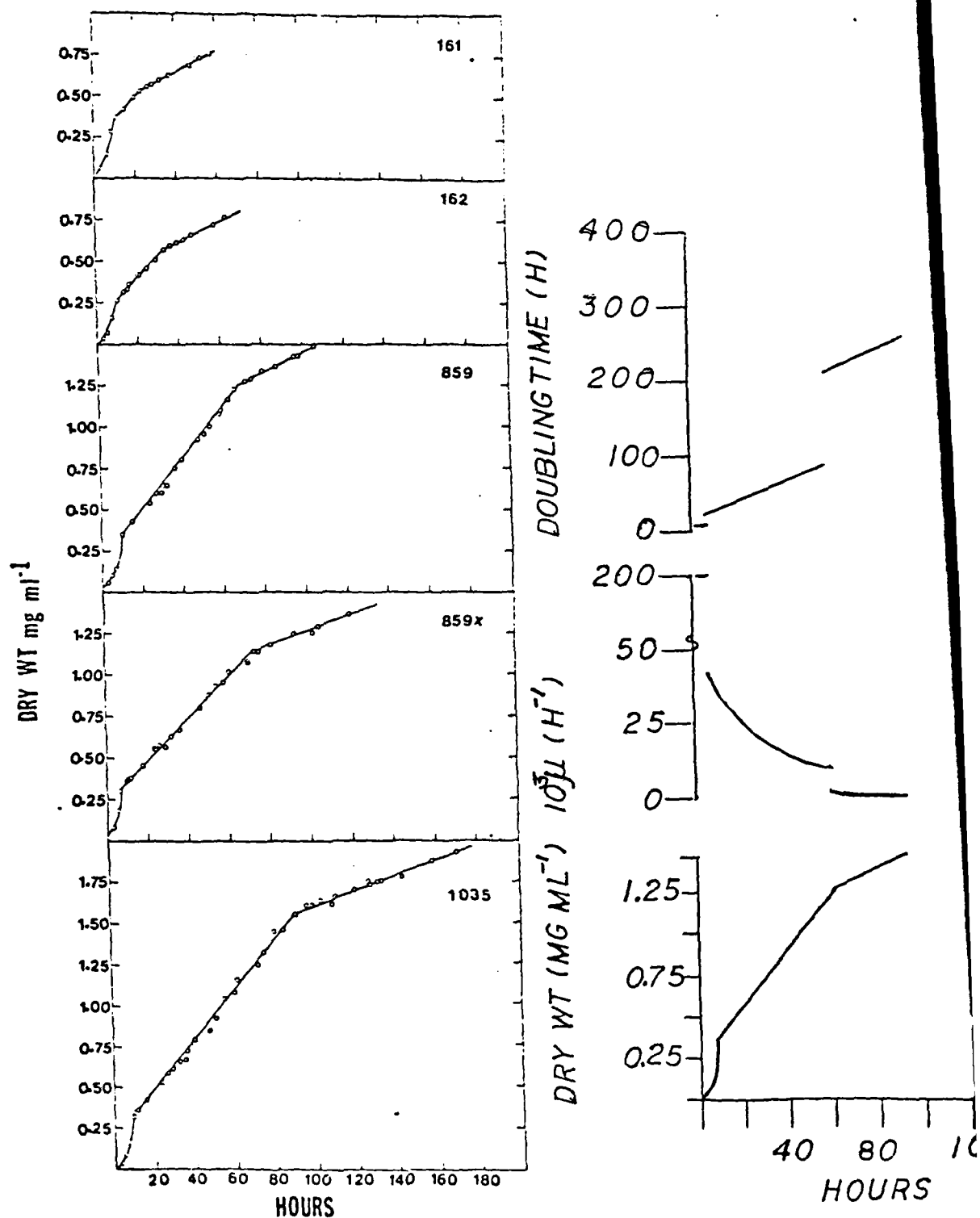


Figure 2. Growth of *E. coli* wild type and *rel* system mutants in the recycling fermentor (left above); NF161 (spot); NF162 (*rel* A1 spot); NF859 (wild type); NF859X (*rel* A1); NF1035 (*rel* A2 *rel* X). The data for NF859 (wild type) is replotted (right above) with calculated doubling times and specific growth rates (μ ; note discontinuity in scale) superimposed.

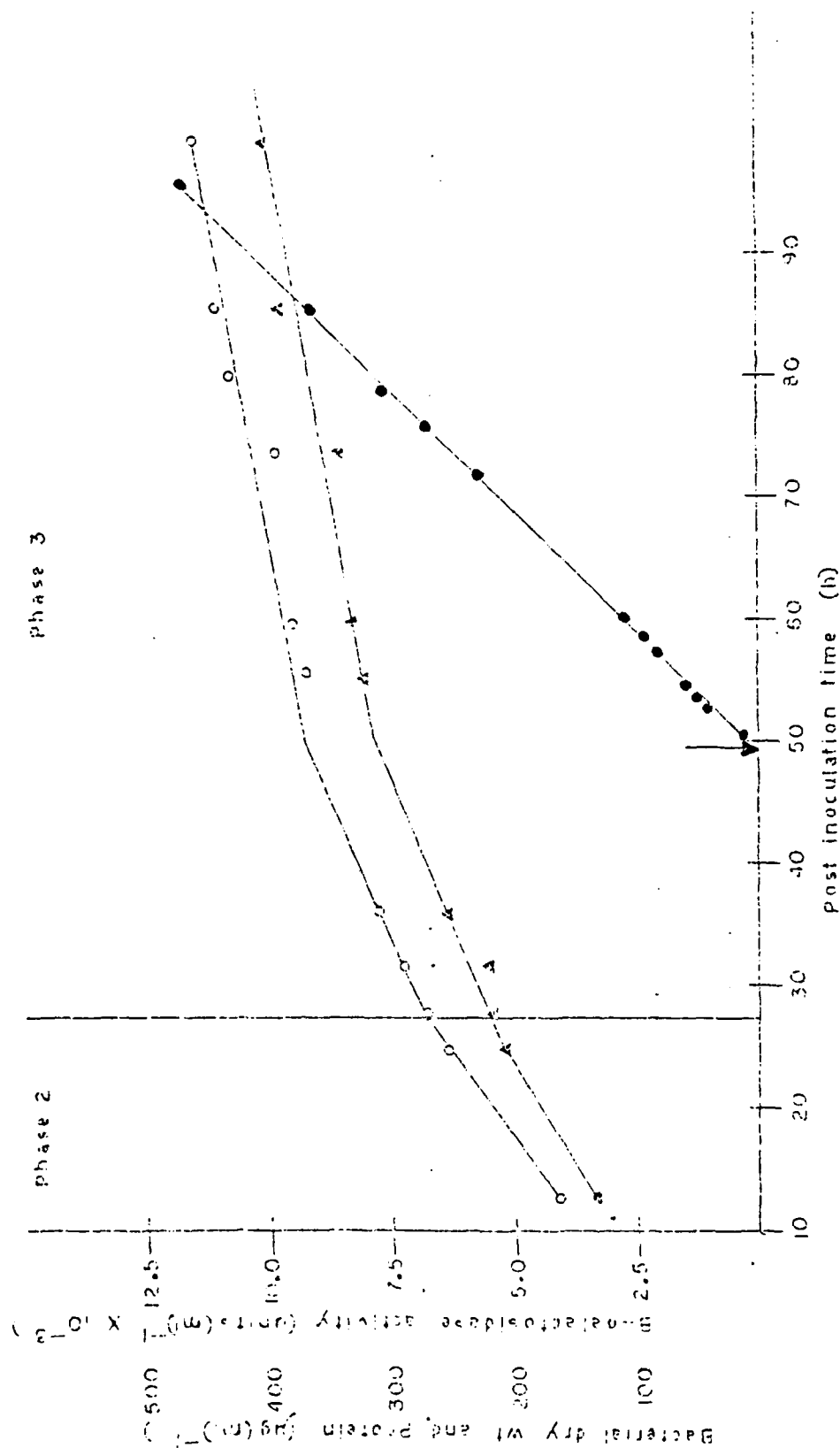


Figure 3. beta galactosidase synthesis in *Escherichia coli* B in the 3rd phase (domain) of growth in the recycling fermentor. The inducer, IPTG, was added continuously to the culture at 0.001M after the time marked at the arrow. Bacterial dry weight (Δ - Δ); bacterial protein (o - o); beta galactosidase activity (● - ●).

Table 1. Growth rate ranges of the three bacterial growth modes.

Doubling Time (T_D) Ranges in hours			
0.3	14	20	80
EXPLOITATION MODE	ADAPTATION (ATTACK) MODE	CONSERVATION MODE	
--nutrient uptake systems saturated	--energy uptake systems unsaturated	--nutrient uptake systems empty or near empty	
--growth rate exponential	--maintenance cost elevated	--maintenance cost maximum	
--maintenance costs minimum	-- $Y \leq 0.6 Y_{max}$	-- $Y \leq 0.2 Y_{max}$	
-- $Y \sim Y_{max}$	--cell cycle stages discrete	--ribosome synthesis interdicted	
--cell cycle stages overlapping	--DNA/RNA/protein synthesis balanced	--peptide elongation slowed	
--DNA/RNA/protein synthesis balanced	--chromosome/membrane/wall extension less coordinate	--chromosome/membrane/wall extension checked	
--chromosome/membrane/wall extension coordinated	--cAMP level maximum	--cAMP level elevated	
	--ppGpp level elevated	--ppGpp level maximum	
	--adaptive functions expressed		
	--endogenous reserves consumed		

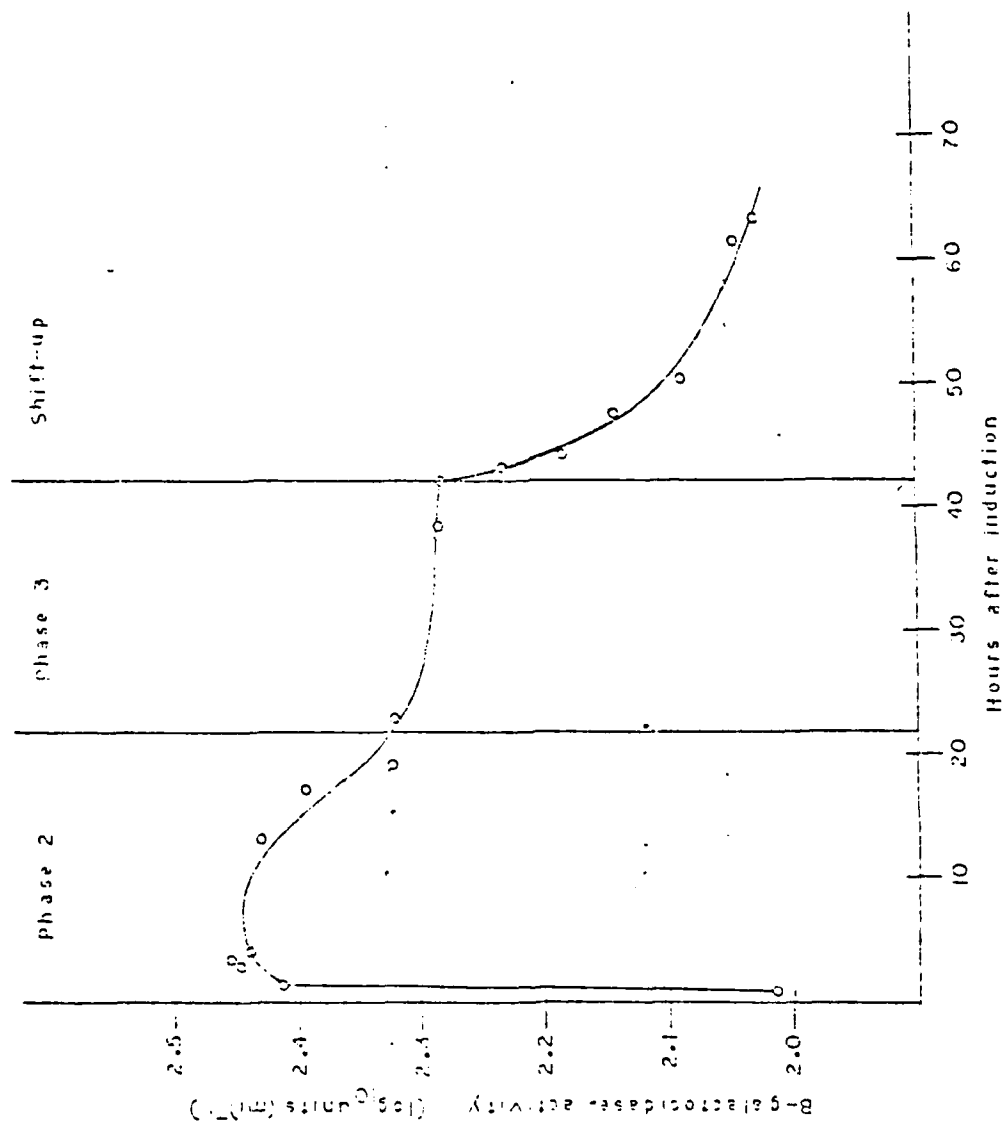


Figure 4. Loss of beta galactosidase from a culture of *Escherichia coli* B induced with a lactose pulse in the 2nd phase during subsequent growth in phases 2 and 3 and a fourfold upshift in the carbon/energy supply rate.

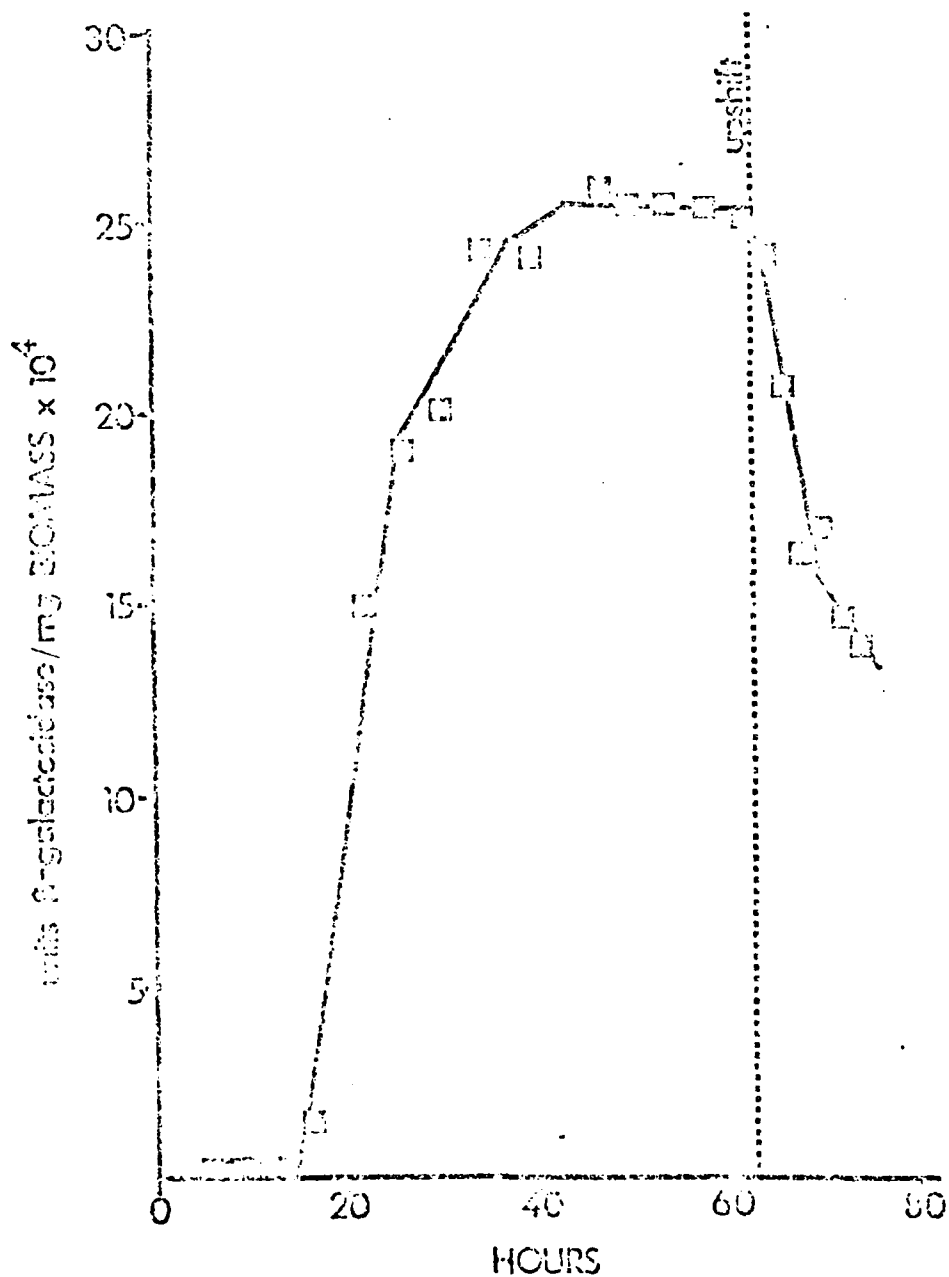


Figure 5. *Escherichia coli* H10407 (enterotoxigenic) growing at pH 8.5 in the recycling fermentor with the influent medium contain 7 mM glucose and 1mM lactose. Phase 2 began at 15 h and phase 3 at 55 h (determined from concurrent estimations of bacterial dry weights).

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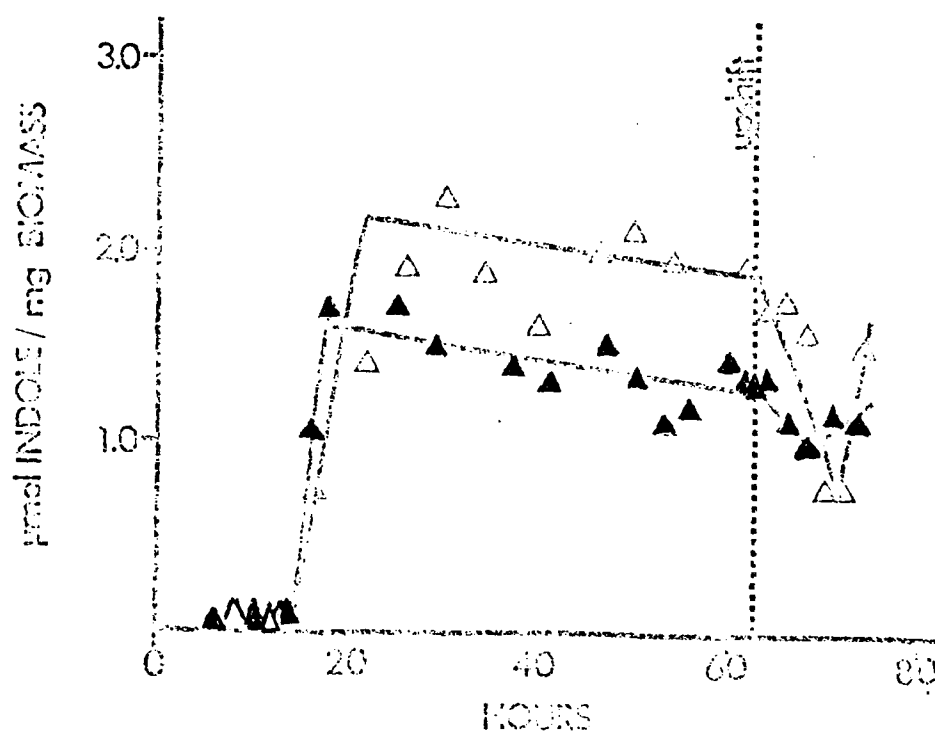


Figure 6. *Escherichia coli* H10407 (enterotoxigenic) growing at pH 8.5₁ in the recycling fermentor with a feed rate of 416 $\mu\text{mol glucose h}^{-1}$ (▲ - ▲) or 833 $\mu\text{mol glucose h}^{-1}$ (Δ - Δ) and upshifted fourfold at the indicated time. The cultures were continuously exposed to 1.5×10^{-3} M L tryptophan. Phase 3 began at 15 h and phase 3 at 55 h (determined from concurrent estimations of bacterial dry weights).

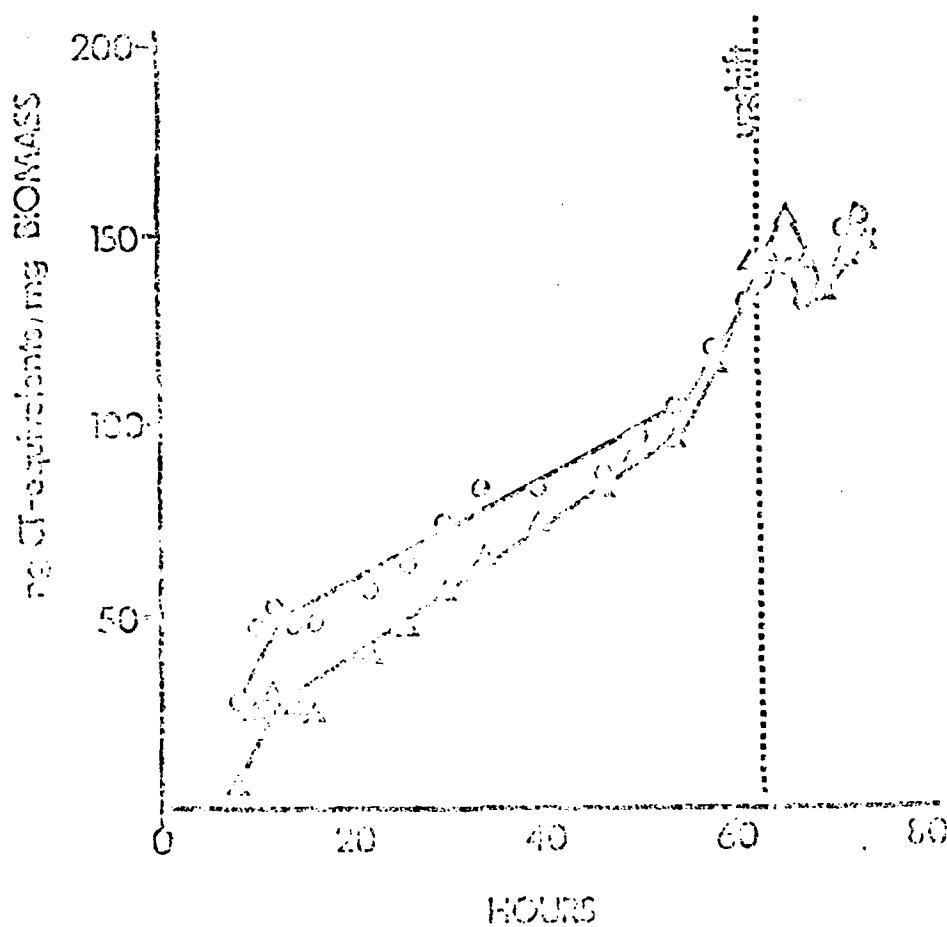


Figure 7. Accumulation of heat labile enterotoxin secreted per mg biomass by *Escherichia coli* H10407 growing at pH 8.5 in the recycling fermentor. The toxin was trypsin-treated (● - ●) for activation in the CHO-cell assay, or untreated (▲ - ▲) to measure spontaneous activation. Phase 2 began at 15 h and phase 3 at 55 h (determined from concurrent estimations of bacterial dry weights).

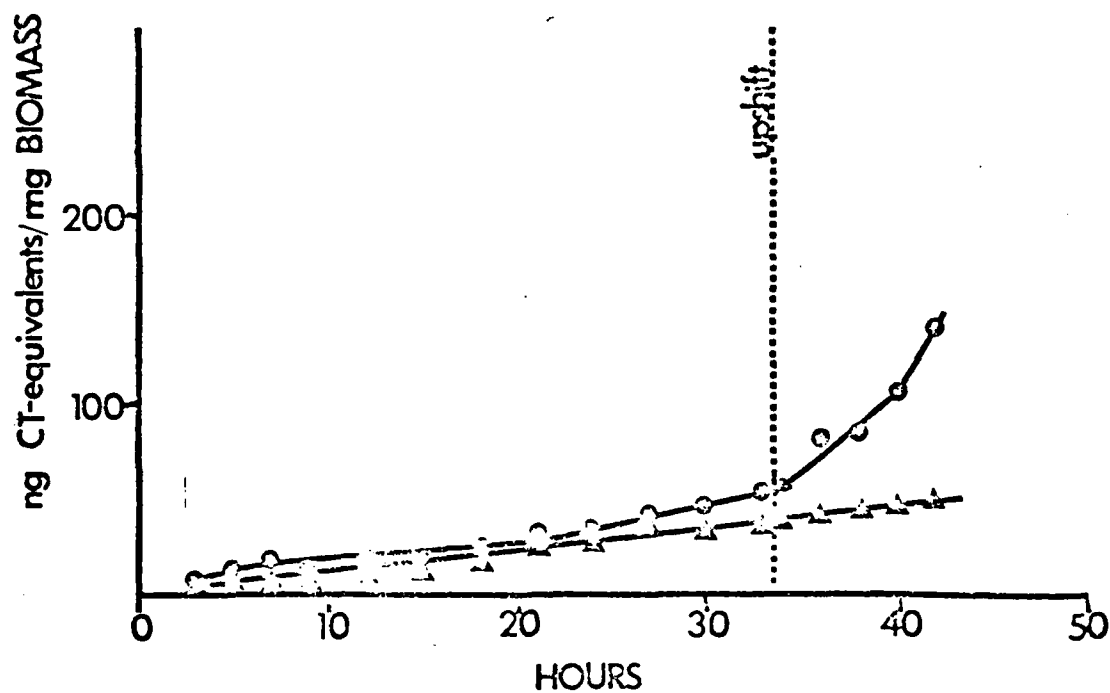


Figure 8. Accumulation of heat labile enterotoxin secreted per mg biomass by *Escherichia coli* 711P307 *crp* growing at pH 8.5 in the recycling fermentor. The toxin was trypsin treated (● - ●) for activation in the CHO cell assay, or untreated to measure spontaneous activation. Phase 2 began at 12 h and phase 3 at 25 h (determined from concurrent estimations of bacterial dry weights).

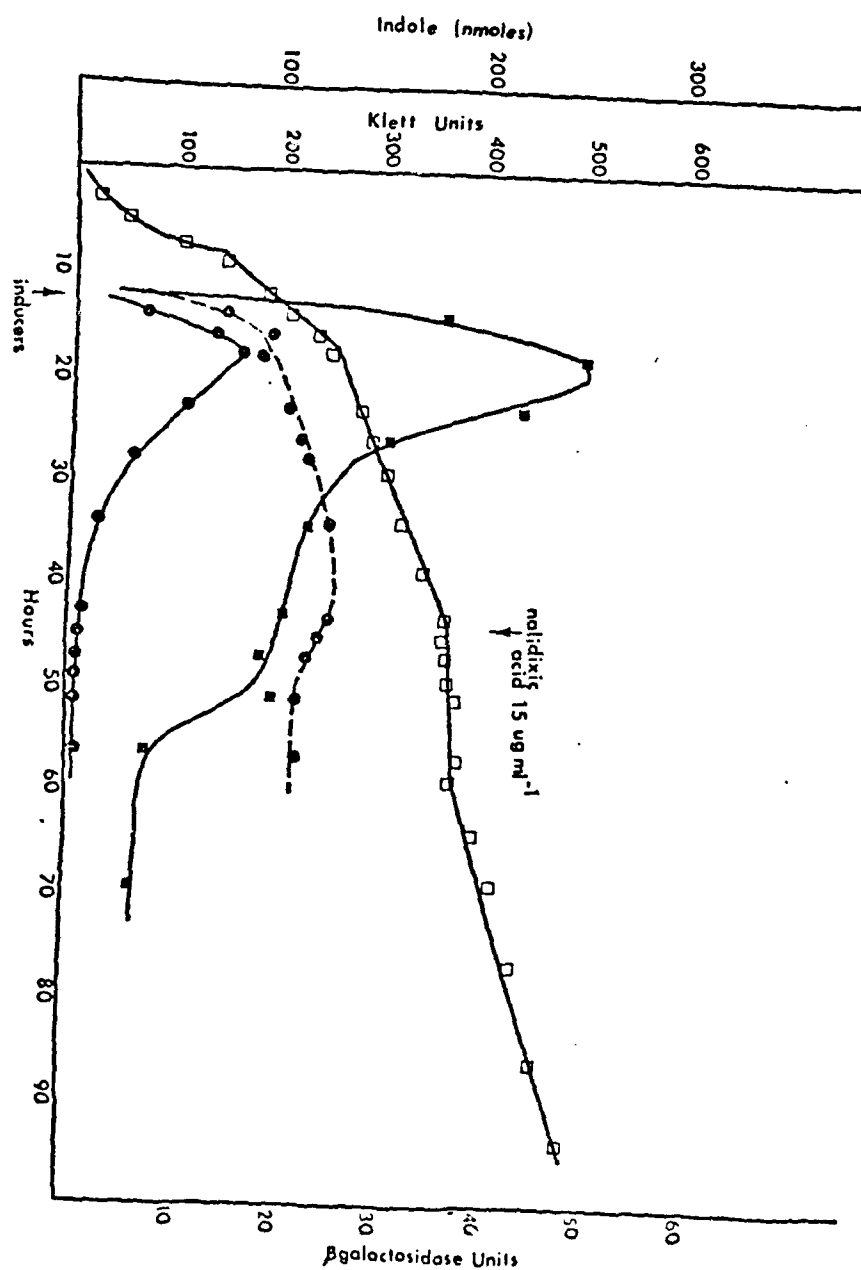


Figure 9. Growth of *Escherichia coli* NF161 (spot1) in the recycling before and after being pulsed with lactose and L tryptophan ($\bullet - \bullet$: filtrate indole; $\bullet - - - \bullet$: tryptophanase activity; $-$ beta galactosidase activity) and nalidixic acid. The phase 1-2 shift at 9 h and the phase 2-3 shift at 18 h can be seen on the curve for bacterial mass ($\square - \square$; Klett units).

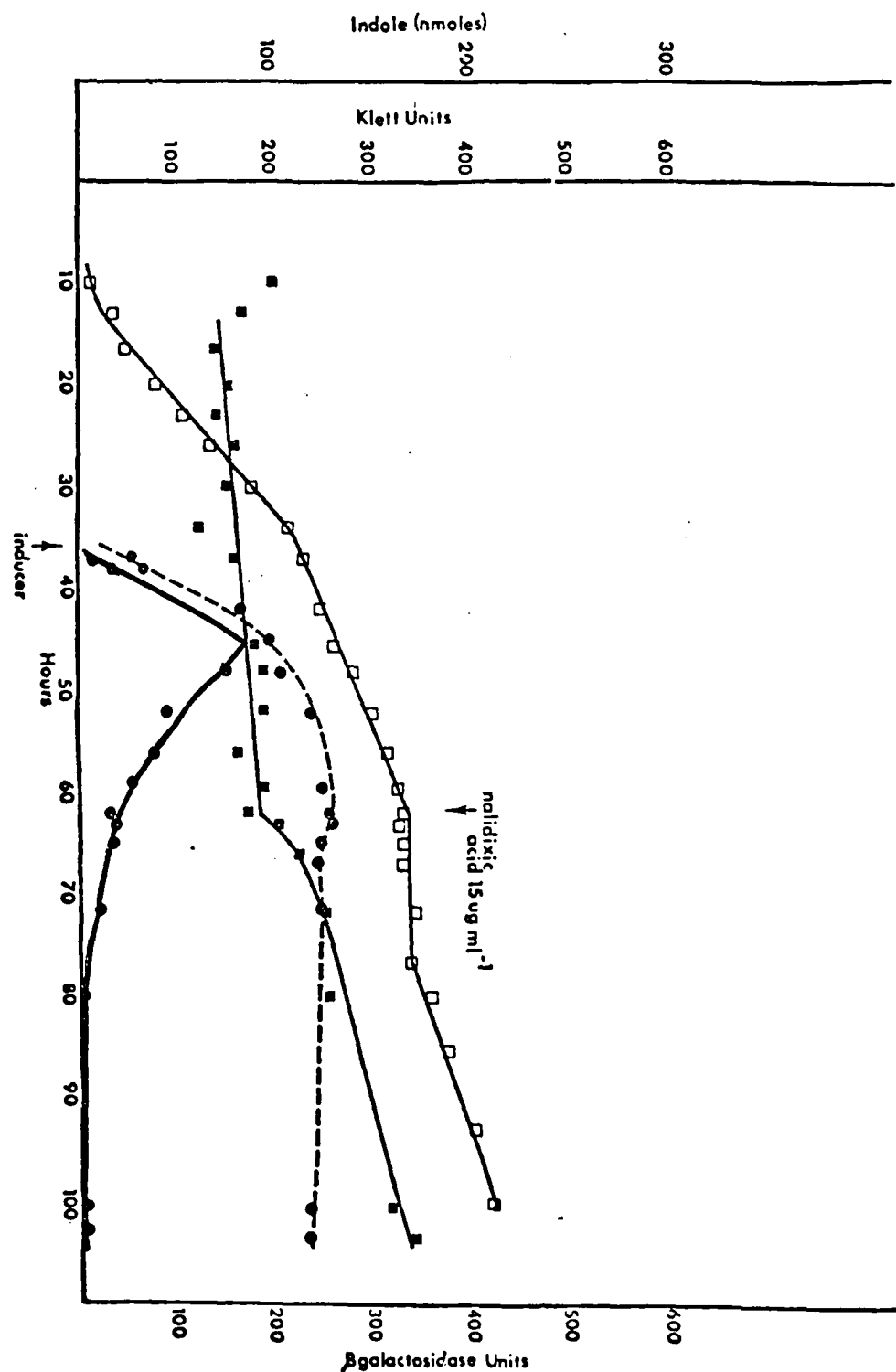


Figure 10. Growth of *Escherichia coli* ATCC33311 in the recycling fermentor after a pulse of L tryptophan (● - ●: filtrate indole; ● --- ●: tryptophanase activity) and nalidixic acid. The beta galactosidase (■ - ■) structural gene is fused to the lambda promoter in this strain. This strain did not show a distinct phase 1-2 shift, but the phase 2-3 shift at 32 h can be seen on the curve for bacterial mass (□ - □): Klett units).

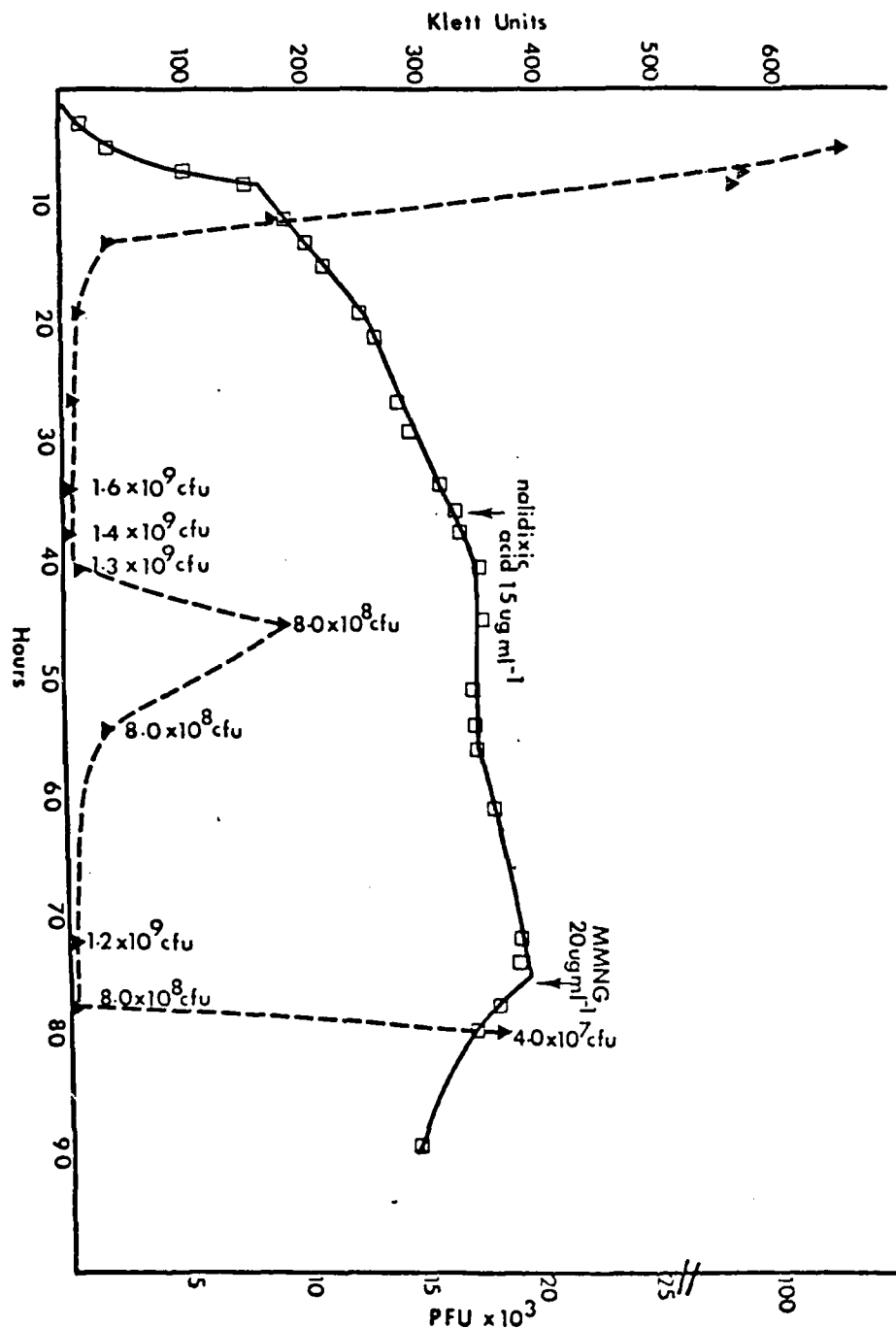


Figure 11. Growth of *Escherichia coli* NF161 (spot11lambda) in the recycling fermentor after a pulse of nalidixic acid and a pulse of MNNG. The phase 1-2 shift at 8 h and the phase 2-3 shift at 18 h can be seen on the curve for bacterial biomass ($\square-\square$). The virus count in the filtrate ($\blacktriangle---\blacktriangle$: pfu) is shown with the viable bacterial count (cfu) suprainposed.

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